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Amperometric Enzyme Electrodes

by

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This was prepared to be published as two chapters in a book entitled "Chemical Sensor Science", reviewing amperometric biosensors. Special emphasis is placed on transport characteristics.

Three generations of amperometric enzyme electrodes have evolved during the past thirty years. The first generation was based on natural enzyme reactions, particularly reduction of oxygen to hydrogen peroxide and amperometric measurement of the oxygen consumed or the hydrogen peroxide formed. These electrodes are in extensive *ex vivo* use today and are being developed for *in vivo* applications in feedback loops for the control of blood glucose levels in diabetics. In the second generation electrodes, the natural oxidant, oxygen, was replaced by oxidizing members of fast redox couples, such as ferricinium or quinone derivatives. These diffused into the substrate-reduced enzymes, oxidized these and were reduced, then diffused out of the enzyme into the solution and then from the solution through a membrane to an electrode where they —

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were reoxidized. The current measured represented the flux of the reduced redox mediators to the electrode. Amperometric biosensors based on the use of diffusing redox couples have been in production since 1987. The newest generation of biosensors is based on electrical "wiring" of enzymes with redox macromolecules, that relay electrons from the substrate-reduced enzymes to the electrodes. The wired enzymes are bound to the surface of electrodes and communicate with these electrically. These electrodes do not require the use of membranes. They are fast, their response times being of one second or less, and are simple to make.

Current research and development devoted to amperometric biosensors emphasize stability, miniaturization, selectivity, sensitivity, reproducibility, manufacturability and cost reduction. Program objectives include the introduction of enzyme electrodes into *ex vivo* compact feedback loops for industrial control and into *in vivo* loops for control of levels of biochemicals in the human body, particularly of glucose levels in diabetics.

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Amperometric Enzyme-Electrode Materials

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III. Sensor Materials

10. Biochemical Sensor Materials

10.4. Enzyme Electrodes

In this section, we address the materials of construction for amperometric enzyme electrodes. The biologically sensitive material is immobilized in intimate contact with the amperometric transducing system and converts the biochemical signal into a quantifiable and processable electrical current. The biological element used for molecular recognition of the target biomolecule can be one of three principal classes: biocatalytic, immunological, or chemoreceptive. For purpose of this overview addressing enzyme-electrode materials, only biocatalytic (enzyme) components will be discussed. Amperometric enzyme-linked immunoassays will not be considered since the principal recognition element in this case is the antigen-antibody immunological interaction. Additionally, our discussion is limited to isolated enzymes (or enzyme plus cofactors), as opposed to whole cells or animal tissue, although the latter contain all the enzymes and cofactors necessary for biocatalytic recognition. Before presenting strategies (and the accompanying materials) for constructing enzyme electrodes, we shall discuss the individual components of an integrated biosensor: the amperometric transducer and the biocatalytic recognition element.

Amperometric Transducer

In electroanalysis by amperometry, a fixed potential difference is maintained between a working (detection/sensing) electrode and a reference electrode placed in the solution that is ionically conductive and contains the substrate. The associated reduction or oxidation at the working electrode is monitored through the current that flows between the working and counterelectrodes. This concept was presented in section 2.2 in the context of an integrated chemical sensor. Here, we discuss only the electrochemical transducer, *i.e.* the electronics of amperometric measurement and the electrode materials commonly used in the fabrication of amperometric detection probes.

Electronics - As compared with the cost of components of other biochemical transducers, the cost of components of amperometric sensors is relatively low. The simple electronic circuitry for measuring the current response of a particular enzyme electrode at a fixed potential requires only a few resistors, a variable resistor (trimmer pot) and a dc power supply (or a fixed potential battery). Somewhat more sophisticated, but still inexpensive circuits are made with these components plus operational amplifiers in the form of simple chips. Of course, such inexpensive circuits are not as versatile as commercially available potentiostats, that enable a wide range of potentials to be scanned; but for sensing applications, the simplest and smallest electronic transducer is sufficient.

Aside from the considerations relating to selectivity, the characteristic that most influences the degree to which a sensor can discriminate between one concentration level and another is the signal to noise ratio. This topic has been discussed for amperometric transducers [1]. Well-understood techniques of grounding and shielding that often reduce noise can be found in electronic texts [2] and are directly applicable. Here we shall discuss only briefly this important topic.

External electromagnetic noise effects can be reduced by good shielding. There are, however, also noise sources that are intrinsic to the transducer, arising either from the electrochemical cell, or the associated electronics. The three major contributors are: the input voltage noise of the current to voltage converter; the impedance noise of the cell; and the area of the working electrode. Careful sensor design, involving electrical and thermal shielding, as well as low-noise electronic components and circuits are essential. While these parameters are dealt with in the design stage of the sensor, the optimum area of the working electrode is best determined empirically by experimentation. In modern systems, a digital recorder/output device is added for data storage, retrieval, and/or feedback control.

Electrodes - In spite of the fact that solid electrodes have been in use for electroanalytical purposes for some time, the selection of the electrode material and its surface preparation are still subject to active research. The working electrodes are usually made of gold, platinum, or some form of carbon (glassy carbon, graphite, reticulated vitreous carbon, carbon paste, fiber or foil). Carbon is favored for enzyme immobilization, because an enzyme can be immobilized on its surface by covalent bonding, adsorption or physical entrapment. In the absence of spurious electroactive species,

platinum electrodes produce substantially lower background currents, and therefore are particularly useful when low limits of detection are required. They are also useful when the electrode process is oxidation of hydrogen peroxide. Additionally, platinum can be aminosilylated to activate the surface for covalent bonding.

The most frequently used electrode material, glassy carbon, often displays complex behavior. Although attempts have been made to formalize and validate surface pretreatment of this material, difficulties arise in obtaining a reproducible response. In general, the preparation of reproducible surfaces is a major limitation of electrodes. This is exemplified by graphite, out of which superior, but not very reproducible, electrodes are made. In this specific case, the best adsorbing and electrocatalytic surface domains are the edges of the lamellae. Neither the surface density nor the chemistry of the edge sites is easy to quantitatively control. When the graphite is porous, these features are even more difficult to control. Although electrode pretreatment procedures can help reproducibility, most "bare" electrodes do not give a reproducible response after extended exposure to protein-containing solutions.

Microelectrodes have become popular in the study of electrochemistry and for amperometric sensing in the past ten years [3]. Because the diffusion zone that surrounds a microelectrode (diameter $< 10\ \mu m$) is spherical, enhanced mass transport to the electrode results and a steady-state current is rapidly achieved after a potential pulse is applied to the electrode. The relatively narrow diffusion zone implies that the faradaic current obtained at microelectrodes is relatively immune to effects of convection in the bulk solution. In flowing streams, the current is independent of flow rate. The decreased capacitance of microelectrodes, coupled with the high mass-transport rates, allows electrochemical measurements in cells with highly resistive solutions.

The choice of the operating potential of the working electrode offers limited selectivity, that can be only slightly improved by dynamic potential modulation. Thus, amperometric sensors often rely on additional chemical layers, in the form of membranes, for enhanced selectivity. Interference by non-enzymatically oxidized species is reduced by covering the electrode of an amperometric device with a membrane that is permeable only to the analyte [4]. Furthermore, membranes also reduce poisoning of

the electrodes by electroactive or surface-active species. The disadvantage of using a membrane is that it forms a physical barrier to the spread of the diffusion layer into the bulk solution, becoming the prime element that limits mass transfer. Because of reduced mass transfer, the limiting current is reduced, *i.e.* the sensitivity is poorer, and the response-time is longer.

Over the past few years, chemically modified electrodes have been used to overcome some of these problems, and this topic will be discussed in more detail in the "System Types" section.

Enzymes

The specificity and sensitivity of an enzyme electrode is determined by the inherent specificity and activity of an enzyme for a given substrate and by the reaction of exposed areas of the base-electrode with spurious substrates. The utilization of an enzyme-catalyzed reaction as the basis for amperometric detection requires that either the cofactor or the products of the substrate-enzyme reaction be electroactive. The enzymes that are useful in amperometric sensors are dehydrogenases and oxidases. The two will be briefly reviewed.

Dehydrogenases - Many dehydrogenases catalyze oxidation/reduction reactions with the aid of nicotinamide containing cofactors (NAD^+/NADH; $NADP^+$/NADPH). These systems have attracted attention because, if a general approach could be derived for biosensors utilizing nicotinamide adenine dinucleotide, NADH, and nicotinamide adenine dinucleotide phosphate, NADPH, dependent dehydrogenases, it would be applicable to sensors for several hundred analytes including ethanol, lactate and pyruvate.

In these dehydrogenases the cofactor is weakly bound to the enzyme and readily dissociates from the apoenzyme. Upon diffusing to the electrode surface, the coenzyme is oxidized or reduced. The oxidation reaction is usually chemically irreversible because a radical intermediate, produced in one-electron oxidation, dimerizes. Reduction of NAD^+ or $NADP^+$ may lead to an inactive dimer or the wrong isomer. Thus, the problem of utilizing these enzymes in amperometric enzyme electrodes is to find catalytic electrode surfaces at which the biological redox reaction will occur cleanly, rapidly and at low overpotentials. Neither radicals that dimerize nor wrong isomers must be produced. Much

of the past work has been directed towards the use of chemically modified electrodes containing redox mediators, among which naphtoquinone derivatives, such as vitamin K_3 appear particularly effective. This approach will be discussed further in the next section.

Another class of dehydrogenases is that of the quinoproteins, containing pyrroloquinolene quinone prosthetic groups. Here, electron-transfer mediators can be used to recycle the quinoprotein, and the reduced mediator can be detected amperometrically.

Flavoprotein Oxidases - The redox centers in oxidases are flavin groups. The flavin prosthetic group, FAD, is tightly bound to the apoenzyme, and the two function electrochemically as an integral unit. Thus, whether the enzyme is in its oxidized or reduced form depends upon the oxidation state of the flavin group. This diagnostically important family of enzymes is particularly suitable for use in enzyme electrodes. A common oxidase/substrate reaction is

$$\text{Substrate} + O_2 \rightarrow H_2O_2 + \text{Product} \quad . \tag{1}$$

Glucose oxidase is the most extensively studied flavoenzyme and will serve to illustrate the various sensor strategies and characterization procedures that are used in these electrodes. In reaction (1), glucose oxidase catalyzes the oxidation of glucose by oxygen to gluconolactone and hydrogen peroxide. Specifically, the flavin redox center, FAD, that is buried deep inside the enzyme glucose oxidase, oxidizes the glucose, while being reduced to $FADH_2$. The electron acceptor, O_2, is then reduced to H_2O_2 and returns the flavin to its original oxidized state (FAD). The detailed reaction scheme is given by:

$$\beta\text{-}D\text{-glucose} + \text{GO·FAD} \rightarrow \text{GO·FADH}_2 + D\text{-glucono-}\delta\text{-lactone} \tag{2}$$

($-2H^+ - 2e^-$ over the full reaction; $+2H^+ + 2e^-$ under the full reaction)

$$O_2 + \text{GO·FADH}_2 \rightarrow \text{GO·FAD} + H_2O_2 \quad , \tag{3}$$

($-2H^+ - 2e^-$ over the right-hand side; $+2H^+ + 2e^-$ under the full reaction)

where GO·FAD represents the oxidized form of the oxidase enzyme and GO·$FADH_2$ the reduced form

of the flavoenzyme conjugate.

Various sensor strategies that are based on this natural oxidation process will be presented in the next section, as well as schemes that utilize synthetic electron acceptors or mediators.

System Types

The first enzyme electrodes [5], [6] were based on the natural glucose oxidase enzymatic reaction, where either the reactant, O_2, or the product of the reaction, H_2O_2, was detected amperometrically. In these sensors, permselective membranes between the sample and the detector electrode are necessary to yield the high specificity required for the analysis of physiological fluids. In a typical configuration, the membrane system comprises three distinct layers. The outer membrane encounters the sample solution and serves to eliminate high molecular weight interfering solutes, such as proteins. The substrate and other small molecules are allowed to enter the second or enzyme layer. The immobilized enzyme catalyzes the conversion of substrate to product, and an electroactive reaction species, such as H_2O_2, is detected amperometrically. Because the electrochemical sensor may be prone to interferences by other small electroactive species, a third (permselective) membrane is required between the enzyme layer and the electrode. Research on such biosensors has resulted in commercially produced systems. An example of a system based on this device is produced by Yellow Springs Instrument Company. The system employs anodic polarography to measure H_2O_2 formed by the lactate or glucose reactions with oxygen, catalyzed by the corresponding oxidoreductase. To prevent interference from spurious electroactive species in blood, a proprietary multilayer membrane that includes a cellulose acetate membrane and a Nucleopore polycarbonate membrane is being used. Although these and other systems work well, their functioning requires the presence of oxygen and their response time is slow (>10 sec).

Enzyme entrapment in electrochemically deposited polymers has also been reported. Because the immobilization procedure only involves the application of a suitable potential to an appropriate aqueous solution of monomers and enzyme, the technique is particularly amenable to the localization of enzymes to small or defined electrode geometries in a controlled manner. Preliminary

studies have shown that glucose oxidase may be incorporated into polypyrrole or poly(N-methylpyrrole) [7] films grown on platinum electrodes, and that the enzyme-catalyzed oxidation of glucose can be followed by the detection of hydrogen peroxide. The stability has not yet matched that of other immobilization techniques.

Signal amplification can be obtained through electrochemical/enzymatic recycling using multiple enzymes, *i.e.*, by utilizing coupled enzyme reactions, where the first enzyme converts the substrate to its product and the second enzyme catalyzes the regeneration of the substrate.

The oxygen dependence of enzyme electrodes can be avoided by replacing the O_2/H_2O_2 couple with diffusing redox mediators, *i.e.*, low molecular weight redox couples with reduction potentials that are thermodynamically favorable for shuttling electrons from the redox center of the enzyme to the surface of the indicator electrode. During the catalytic cycle, the oxidized enzyme is reduced by its substrate, and is then preferentially reoxidized by the mediator, not by O_2. The reduced mediator then diffuses to the electrode surface where it is oxidized via a heterogeneous charge-transfer reaction. The generalized catalytic reaction scheme is as follows:

$$\text{Substrate} + \text{E·FAD} \rightarrow \text{E·FADH}_2 + \text{Product} \tag{4}$$

$$2\,M_{ox} + \text{E·FADH}_2 \rightarrow \text{E·FAD} + 2\,M_{red} + 2\,H^+ \tag{5}$$

$$M_{red} \rightarrow M_{ox} + e^- \ . \tag{6}$$

Again, FAD represents a flavin redox center within the enzyme, E, and the mediator M_{ox}/M_{red} is assumed to be a one-electron transfer couple. Reaction (6) will be thermodynamically favored if the standard reduction potential of the electron accepting mediator is positive of that of the enzyme-bound flavin. The redox potential of glucose oxidase is, at pH 7.2, near -340 mV (SCE), but the redox potentials of enzyme-bound flavins can vary with their protein environment by as much as 300 mV and are pH dependent. Another advantage of employing mediators is that electron shuttles can be selected with redox potentials in a range less anodic (+100 to + 400 mV *vs* SCE) than those required to oxidize hydrogen peroxide. These sensors should be less susceptible to interferences from blood components.

A number of different redox mediators can be used for the oxidation of flavoprotein enzymes [8]. Silverman and colleagues [9] in 1964 first described methylene blue as a mediator for the oxidation of flavoprotein enzymes. Methylene blue, which oxidizes $FADH_2$ to FAD, is simultaneously reduced to (leuco) methylene blue, that is then reoxidized at an electrode. At conducting organic salt electrodes, *e.g.*, those formed from the N-methylphenazinium (NMP^+) or tetrathiafulvalinium (TTF^+) cation and the tetracyanoquinodimethane anion ($TCNQ^-$), electron transfer from glucose oxidase and other enzymes is rapid [10].

Cass *et al.* [11] introduced in 1984 metalorganic mediators and widely tailored their properties with respect to charge and potential. They constructed a group of amperometric electrodes for the mediated oxidation of glucose oxidase based on derivatives of the ferrocene/ferricinium redox couple. An important family of redox mediators based on quinones and quinone derivatives was developed by Senda *et al.* [12]; others involve ruthenium complexes, such as hexammines developed by Crumbliss *et al.* [13]; and octacyano-molybdates and octacyanotungstates developed by Taniguchi *et al.* [14].

The most recent amperometric enzyme electrodes do not require either O_2 or a diffusing redox mediator for their operation. Electron transfer from the redox centers of the enzymes to the electrodes takes place through electron relays attached to the protein of the enzymes. Three types of electrodes have been made. In the first, electron relays, that are fast redox couples of redox potentials oxidizing with respect to the enzymes, are covalently or coordinatively bound to the enzyme-proteins. Examples include ferricinium/ferrocene carboxylates bound to enzyme-amines through amide links [15], [16]. In the second type, a segment of a polycationic redox polymer is adsorbed on an electrode, then electrostatically complexed and/or covalently bound to the polyanionic enzyme, to form a thin "wired" enzyme film. The transfer of electrons is from the substrate reduced $FADH_2$ enzyme centers, via the redox centers of the polymers, to the electrode. Examples of redox polymers include $[Os(bpy)_2Cl]^{2+/3+}$ complexes of partially quaternized poly (vinyl pyridine) and water soluble copolymers of vinyl ferricinium/ferrocene and vinyl pyridinium chloride. The two latter polymers form electrostatic complexes with negatively charged enzymes. Covalent bonds to the enzymes can be added by copolymerizing vinyl pyridine and 4-aminostyrene, forming the $[Os(bpy)_2Cl]^{2+/3+}$ complex

with the polymer, quaternizing the residual uncomplexed pyridine rings, diazotizing the aniline functions of the polymer and then coupling the diazonium ions to enzyme-tyrosines [17]. Such covalen. bonding prevents excessive coiling of polycations at high ionic strength and thus reduces the variation of the current with ionic strength [18]. In three dimensional networks of redox polymers that bind covalently a large number of enzyme molecules and "wire" these to the electrode, high current densities have been reported [19]. Their wiring networks also contain $[Os(bpy)_2Cl]^{2+/3+}$ redox centers complexed to chain-pyridine rings, but the polymer is now made with cross-linkable functions. Mass-transport limited current densities of 0.5 mA cm^{-2} are reached at 30 mM glucose concentration when such sensors are made with glucose oxidase.

Summary

Three generations of amperometric enzyme electrodes have evolved during the past thirty years. The first generation was based on natural enzyme reactions, particularly reduction of oxygen to hydrogen peroxide and amperometric measurement of the oxygen consumed or the hydrogen peroxide formed. These electrodes are in extensive *ex vivo* use today and are being developed for *in vivo* applications in feedback loops for the control of blood glucose levels in diabetics. In the second generation electrodes, the natural oxidant, oxygen, was replaced by oxidizing members of fast redox couples, such as ferricinium or quinone derivatives. These diffused into the substrate-reduced enzymes, oxidized these and were reduced, then diffused out of the enzyme into the solution and then from the solution through a membrane to an electrode where they were reoxidized. The current measured represented the flux of the reduced redox mediators to the electrode. Amperometric biosensors based on the use of diffusing redox couples have been in production since 1987. The newest generation of biosensors is based on electrical "wiring" of enzymes with redox macromolecules, that relay electrons from the substrate-reduced enzymes to the electrodes. The wired enzymes are bound to the surface of electrodes and communicate with these electrically. These electrodes do not require the use of membranes. They are fast, their response times being of one second or less, and are simple to make.

Current research and development devoted to amperometric biosensors emphasize stability, miniaturization, selectivity, sensitivity, reproducibility, manufacturability and cost reduction. Program objectives include the development of new materials that meet the above requirements, as well as be biocompatible.

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Principles of Amperometric Enzyme Electrodes

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II. Fundamental Sensor Principles

5. Biochemical Phenomena

5.3. Principles of Amperometric Enzyme Electrodes

An amperometric electrode records current flow in an electrochemical cell at an applied potential, and the combination of an amperometric device with an immobilized redox enzyme provides a highly selective and sensitive method for the detection of a given biomolecule. In such an enzyme electrode, the rate of substrate reaction is transduced into a current. The electrons transferred between the substrate and the enzyme are intercepted and are then transferred either through a mediator or directly to (or from) an electrode. Alternatively, the concentration of a reactant, e.g. O_2, is assayed amperometrically. Transfer of electrons is often mediated by an electroactive species. The mediating electroactive species must be selected so as to be oxidized or reduced at the electrode surface at a diffusion-controlled rate, if the corresponding steady-state faradaic current is to be a direct measure of the extent of the enzyme catalyzed reaction. Thus, when the detection electrode is set at the appropriate constant applied potential relative to a reference electrode and in the absence of spurious electroactive species, a plot of the recorded diffusion-limited current, i_{lim}, versus concentration of substrate, c_S, is linear over a limited range of concentration. The resulting calibration curve (commonly, $i_{lim} = \text{constant} \cdot c_S$) can then be used for the determination of an unknown substrate concentration.

In this section, mathematical models of amperometric enzyme electrodes are presented. They are categorized according to the mechanism for electron-transfer between the redox enzyme and the electrode surface and are designated as first, second, and third generation. First generation sensors are based on the natural oxidase enzymatic reaction, where either the reactant, O_2, or the product of the reaction, H_2O_2, is detected amperometrically. Second generation sensors utilize synthetic, diffusing redox mediators, and third generation devices are based on either nondiffusing mediators or no mediator at all. Finally, the objective of the section is to provide starting scientists and engineers with an overall working knowledge of the underlying theoretical principles that govern amperometric enzyme electrodes.

First Generation Sensors

We review the theoretical work that has been carried out to analyze first generation amperometric enzyme electrodes. The mathematical models to be presented are useful for sensor characterization, fundamental parameter determination, and ultimately, sensor design and optimization. Before discussing specific model results, we give a general description of the transport and kinetic processes accounted for in the models and state the assumptions that are used.

Model Description - A schematic of a "typical" first generation substrate sensing device is given in figure 1, where a simple enzyme electrode geometry is assumed for the purpose of modeling. A homogeneous membrane of thickness L, containing uniformly distributed immobilized (oxidase) enzyme, is placed directly adjacent to the surface of the amperometric product (H_2O_2) or cosubstrate (O_2) sensitive electrode. The corresponding electrode reactions depend on the mode of operation of the sensor (reduction of O_2 at the cathode or oxidation of H_2O_2 at the anode), as well as the enzyme catalyzed substrate-cosubstrate reaction. Each will be further discussed later. The diffusion boundary layer δ in the solution immediately adjacent to the membrane depends on the stirring conditions in the bulk solution. In this one-dimensional problem, the origin is taken as the membrane-electrode interface, and z is the perpendicular or axial distance from the electrode.

Actual enzyme electrodes often have a multilaminate, rather than homogeneous, membrane structure and therefore differ slightly from the geometry described here. Also, it is sometimes advantageous to incorporate a membrane over the catalytic layer. These additional diffusional resistances will not be considered in this paper to retain simplicity, but can be readily introduced as series resistances in the final model formulation. Practical enzyme electrodes may also contain multiple enzymes homogeneously dispersed in the catalytic layer, so that the overall process abridges a series of reactions. Electrodes of this type have been modeled and will be described later.

Figure 1 illustrates the coupled kinetic and transport processes that play a role in amperometric enzyme-electrode measurements. Although numerous and quite different strategies for sensor operation are possible, the physicochemical phenomena that can limit the overall detection

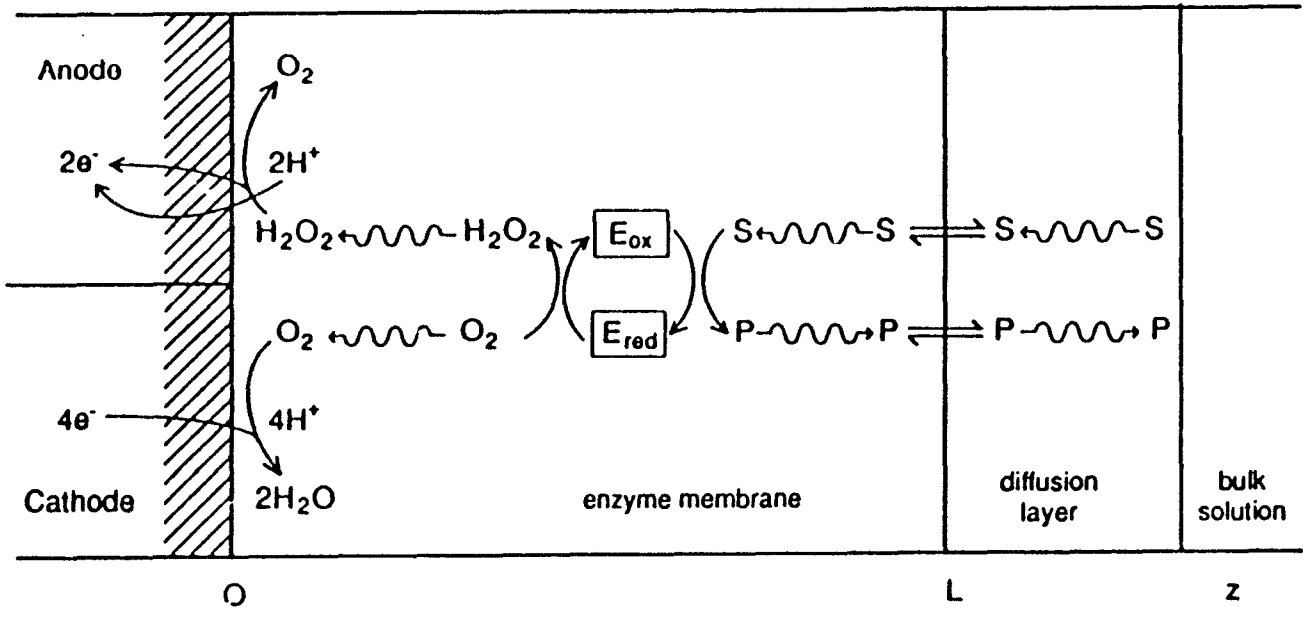

Figure 1. First generation amperometric enzyme electrode. ☐ represents an entrapped enzyme site within the homogeneous membrane. The symbols ⟶, ⇝, ⇌ denote heterogeneous or homogeneous reactions, mass transfer, and species partitioning, respectively.

process are generally the same. Possible limitations are solution-phase "external" and membrane-phase "internal" mass transfer, denoted in the figure by the symbol $-\sim\!\!\!\!\!\!\rightarrow$. The solutes may exhibit differential solubility between the membrane and solution phases and partition accordingly. The species partitioning is shown with $\rightleftharpoons$, indicating an equilibrium process as opposed to being kinetically hindered. Additionally, kinetics of the homogeneous enzyme reaction or the heterogeneous electrode reactions can control the overall process.

Governing Equations - Before characterizing the specific types of enzyme electrodes, we discuss the governing equations that describe the kinetic and transport processes in electrolytic solutions as they apply to the bioanalytical sensor shown in figure 1. Assuming no convective flow within the membrane containing the immobilized enzyme and that the effects of ionic migration are negligible due to excess supporting electrolyte, mass transfer of the reacting species will be primarily due to diffusion. Thus, the steady-state material balance equation for species *i* is given by

$$D_i \frac{d^2 c_i}{dz^2} = \nu_{i,l} \hat{R}_l \quad , \tag{1}$$

where D_i is the diffusion coefficient. $\hat{R}_l$ is the rate of the homogeneous reaction l, and the stoichiometric coefficient $\nu_{i,l}$ is positive for reactants and negative for products in the enzyme catalyzed reaction. This commonly used diffusion-reaction equation can be solved to determine the concentration distribution of species i (*i. e.* the substrate, O_2 and H_2O_2) once the appropriate boundary conditions and the kinetics of the homogeneous reaction are specified.

Assuming that the kinetics of the immobilized (oxidase) enzyme reaction given in figure 1 are of the form corresponding to a two-substrate ping-pong reaction, the reaction rate per unit volume is given by the following expression [1]

$$\hat{R} = \frac{V_{max} c_S c_{O_2}}{K_S c_{O_2} + K_{O_2} c_S + c_S c_{O_2}} \tag{2}$$

where V_{max} is the maximum velocity of the reaction per unit volume and K_S and K_{O_2} are the Michaelis constants for the substrate and dioxygen cosubstrate, respectively. This rate equation is assumed to be independent of the concentration of the products and also can be readily reduced to the analogous

one-substrate Michaelis-Menten expression by letting the concentration of the cosubstrate become relatively large.

The final requirement for the solution of the material balance equation (1) for the three solutes (i = S, O_2 and H_2O_2) is the specification of the boundary conditions at the electrode-membrane interface and the membrane-solution interface. The operating potentials and surface conditions constituting the electrode boundary conditions also are useful for classifying first generation sensors. In figure 1, the possible electrode reactions are summarized for the two types of amperometric enzyme electrodes. The first, a cosubstrate-sensitive electrode, monitors the enzyme reaction by electrochemically measuring dioxygen reduction using a platinum cathode (or Clark oxygen electrode). The second, a product-sensitive electrode, employs a Pt anode to monitor hydrogen peroxide production, a measure of the amount of substrate present in the solution.

At potentials between −500 and −700 mV *versus* an Ag/AgCl electrode (hereafter, the cathodic operating conditions) oxygen is reduced by the following two step process

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \qquad (3)$$

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \ , \qquad (4)$$

where both O_2 and H_2O_2 are electrochemically active. However, the boundary condition is simplified if the following overall four electron-transfer reduction is assumed:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \ . \qquad (5)$$

At electrode potentials between +600 and +700 mV *vs.* Ag/AgCl (the anodic operating conditions) only H_2O_2 is active and is consumed via the two electron-transfer oxidation reaction

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \ . \qquad (6)$$

At these potentials, the electroactive solute (O_2 or H_2O_2) is consumed rapidly at the electrode (cathode or anode) surface, with the result that the reactant concentration at the membrane-electrode interface is maintained at zero, and the flux to the electrode, *i.e.*, the current, is proportional to the substrate concentration. Glucose, a particular substrate of interest, does not react significantly at a shiny Pt electrode surface at these potentials. Thus, the boundary conditions at the membrane-electrode

interface are:

under both operating conditions,

$$\frac{dc_S}{dz} = 0; \quad z = 0 \tag{7}$$

under the cathodic operating conditions [reaction (5)],

$$\frac{dc_{H_2O_2}}{dz} = 0; \quad z = 0 \tag{8}$$

$$c_{O_2} = 0 \quad \text{for} \quad i = i_{lim}; \quad z = 0 \tag{9}$$

and under the anodic operating conditions,

$$\frac{dc_{O_2}}{dz} = 0; \quad z = 0 \tag{10}$$

$$c_{H_2O_2} = 0 \quad \text{for} \quad i = i_{lim}; \quad z = 0 \tag{11}$$

The resulting current density under cathodic operating conditions is given by:

$$i_c = 4F D_{O_2} \left[\frac{dc_{O_2}}{dz} \right]_{z=0} \tag{12}$$

and under anodic operating conditions is

$$i_a = 2F D_{H_2O_2} \left[\frac{dc_{H_2O_2}}{dz} \right]_{z=0} \tag{13}$$

This completes the mathematical statement of the problem and introduces our review of the theoretical work that has been reported in the literature to characterize bioanalytical sensors.

Model Results - Modeling results for first generation amperometric enzyme electrodes are presented next. Carr and Bowers [1] have reviewed the theoretical work that has been done to characterize amperometric enzyme electrodes, including the work of Mell and Maloy [2], who first developed a mathematical model for both the steady-state and the transient response of an amperometric glucose-sensitive electrode accounting for internal mass transfer and enzymatic reaction kinetics. The discussion of their digitally simulated results therefore is brief. Mell and Maloy concluded that high values of the internal substrate modulus $\sigma_S^2 = V_{max} L^2 / D_S \cdot K_S$ (> 10), *i. e.* when the concentration of the immobilized enzyme is high or the diffusional resistance is substantial, improve the sensitivity to

glucose concentration by extending the linear operating range to concentrations greater than K_S.

Bartlett and Whitaker [3] modeled the steady-state behavior of an amperometric product-sensitive electrode similar to that in reference [2]. The model accounts for substrate and product diffusion and an immobilized enzymatic reaction, within a conducting polymer film, as well as substrate partitioning. A rate expression describing the enzyme catalyzed reaction mechanism illustrated in figure 1 is used that employs the quasi-steady-state assumption. The resulting equation is similar to the ping-pong expression given by equation (2) (although different notation is used). The cosubstrate is assumed to be in excess throughout the polymer film, and its concentration therefore is constant. Analytical solutions are obtained by linearizing the substrate-cosubstrate enzyme-catalyzed reaction-rate expression.

In the limiting case (case B), the cosubstrate substrate is taken to be large so that regeneration of the reduced enzyme by reaction with O_2 is not limiting and $K_S \gg c_S$ is assumed [$K_S c_{O_2} \gg (K_{O_2} + c_{O_2}) c_S$ in our notation]. This is the same as linearizing equation (2) in Mell and Maloy's analysis such that the first order reaction rate is given by $\hat{R} = V_{max} c_S / K_S$ and V_{max} / K_S is the apparent first order rate constant. The resulting current density is given by (equation 19 in reference [3])

$$i_a = \frac{2 F D_S \alpha_S c_{S,\infty}}{L} \left[1 - sech \left[\frac{L}{\hat{\delta}} \right] \right] , \tag{14}$$

where $\hat{\delta} = \sqrt{D_S K_S / V_{max}}$ is the enzyme reaction penetration depth (the distance over which the substrate can diffuse into the film before undergoing reaction). When the film is thin relative to the reaction zone ($L \ll \hat{\delta}$), the current density reduces to $i_a = F L V_{max} \alpha_S c_{S,\infty} / K_S$. Under these conditions of enzyme reaction control ($\sigma_S^2 < 0.1$), the entire membrane is a uniform reaction zone. Thus, the current increases by increasing the film thickness L or the enzyme loading c_E. When the film is thick ($L \gg \hat{\delta}$) relative to a small reaction zone near the solution-membrane interface, the current density reduces to $i_a = 2 F D_S \alpha_S c_{S,\infty} / L$ and is independent of enzyme loading. Thus, the response of the enzyme detection electrode is not affected by changes in the enzyme activity during use or storage. This optimum condition results when the enzyme reaction is fast ($\sigma_S^2 > 10$) or the system is controlled

by internal diffusion.

Probably the most comprehensive theoretical treatments of first generation amperometric enzyme electrodes under steady-state conditions have been carried out by Gough, Leypoldt, and Tse [4], [5], [6], [7], and we shall summarize their findings. The previous attempts at modeling first generation devices have considered only one substrate or pseudo one-substrate reactions, and the general approach has been to either obtain solutions to the simplified linearized equations [3] or solve the nonlinear equations numerically [2]. Gough and Leypoldt in reference [4] classify the sensor types according to common design features and give a thorough physical description of each before presenting the details of the mathematical aspects of the various enzyme electrodes. The classification system includes:

- one-substrate product-sensitive electrodes
- two-substrate product-sensitive electrodes
- two-substrate cosubstrate-sensitive electrodes

For each of these categories, both steady-state potentiometric and amperometric enzyme electrodes are discussed. This approach is more complete than other modeling efforts, at once taking into account the effects of partitioning, internal and external diffusional resistances, and nonlinear reaction kinetics. Additionally, the results are expressed in a simple, analytical form that is convenient for sensor design.

The developed model of Gough and Leypoldt in reference [4] is similar to the product-sensitive electrode model of Bartlett and Whitaker [3], but the boundary condition at the membrane-solution interface is more general and accounts for external diffusion. For large values of the Thiele modulus, ϕ^2 (the ratio of the potential rate of reaction to the potential rate of diffusion), the perturbation analysis yields an analytic solution for the current density without having to linearize the enzyme reaction-rate expression $\hat{R}(c_S)$, as was done in reference [3]. The current, given by

$$i_a = \frac{2F D_S \alpha_S c_{S,\infty} \Psi}{(1 + Bi_P^{-1})L} , \tag{15}$$

is a function of the bulk substrate concentration $c_{S,\infty}$ and the parameter

$$\Psi = 1 - \eta\phi^2 \left[\frac{1}{Bi_S} - \frac{1}{Bi_P} \right] , \qquad (16)$$

where η is the effectiveness factor introduced by Aris [8] for diffusion-reaction systems. The effectiveness factor is a measure of the actual average total reaction rate within the membrane divided by the rate that would prevail in the absence of any diffusional efforts. $1/Bi_i$ represents the external diffusional resistance of species i (substrate or product). Thus, the parameter Ψ indicates the extent to which the sensor response deviates from linearity due to internal and external transport resistances. The model suggests that the presence of significant external boundary-layer resistances can affect the linearity of enzyme calibration curves, although slightly, and that this effect becomes greater with increasing concentration of the immobilized enzyme (or small values of the important parameter Bi_S/σ_S) and where the substrate and product differ substantially in mass-transfer characteristics (or $Bi_S/Bi_P \neq 1$).

Although details of the mathematical analysis are given [4] for only a one-substrate product-sensitive electrode, the same procedure can be applied to two-substrate enzyme sensors (using either a product or cosubstrate-sensitive electrode) and the perturbation analysis yields an explicit solution for the substrate concentration for the special case of large Thiele modulus or relative activity σ_S. In their succeeding paper, Leypoldt and Gough [5] presented a more general model of the cosubstrate (O_2)-sensitive electrode accounting for any value of σ_S. This type of electrode employs a different sensing mechanism from the product (H_2O_2)-sensitive electrodes; it measures a difference between the cosubstrate flux (or current) in the absence of the enzymatic reaction and when the reaction is proceeding. Therefore, the definition of the glucose difference current is mathematically different from the current expression for a product-sensitive electrode, but the starting point for the analysis, the principle of conservation of mass, remains the same. Again, the steady-state governing equation (2) is applicable for describing the reaction and diffusion processes in the membrane for species $i = S$ and O_2 (as opposed to $i = S$ and H_2O_2 for the product-sensitive electrode). The equations of the two-substrate enzyme electrode form a non-linear boundary value problem that requires numerical solution. The effects of varying the relative catalytic activity on the sensor response to glucose were

predicted. However, when the cosubstrate (O_2) is present in excess throughout the catalytic membrane, the more simple one-substrate model [2], [3], [4] should be entirely adequate. When oxygen is not present in excess, the two-substrate model predictions can differ notably from those of a one-substrate model. The two-substrate model predicts a linear response for membranes of high relative catalytic activity (similar to the one-substrate model) as long as the glucose concentration in the membrane is low relative to the oxygen concentration. As the substrate concentration increases, the system becomes limited by O_2, and glucose assay is not possible. Interestingly, the two-substrate model predicts that glucose sensitivity is possible (although the signal is nonlinear over a broad concentration range) for membranes of lower relative catalytic activity. This results from less O_2 consumption within the membrane because the reaction rate is lower for smaller σ_s. Thus, it may be advantageous to decrease the enzyme loading as a means of extending the range of sensitivity to glucose in the presence of otherwise limiting oxygen concentrations. Again, these results are unique to the two-substrate enzyme-electrode model because the one-substrate model (for no O_2 limitations) predicts that the detectable range increases with increasing immobilized enzyme activity. Finally, the two-substrate model of Leypoldt and Gough [5] was used to predict the effects of the mass-transfer parameters, Bi_S and Bi_{O_2}, on the glucose-electrode response. When the relative catalytic activity is low, variations in the external mass transfer conditions, as specified by the respective mass transport parameters, have little effect, since the system is dominated by reaction kinetics. However, when the relative catalytic activity is high, a change in external mass transfer conditions can play a significant role.

Gough *et al.*, using an approach similar to their previous modeling efforts [4], [5], developed a more general and complex model [6] of a glucose electrode, where glucose oxidase and catalase are coimmobilized at comparable catalytic activities in a homogeneous membrane adjacent to a rotating disk electrode. The reaction diffusion equation (2) for all three solutes (i = S, O_2, and H_2O_2) forms a system of coupled nonlinear ordinary differential equations that must be solved numerically with the appropriate boundary conditions. Under cathodic conditions, both oxygen and hydrogen peroxide may contribute to the total current when excess catalase is not present in the membrane. Therefore, the

total (cathodic) current is given by the sum of equations (12) and (13). Four cases were investigated, corresponding to specific combinations of immobilized enzymes present in the membrane. Finally, kinetic parameters corresponding to the intrinsic maximal velocity and Michaelis constants of the immobilized enzymes were estimated by regression analysis of data based on an appropriate two- or three- parameter model. It was found that immobilization reduced the maximal intrinsic velocity, but had no detectable effect on the Michaelis constants. In all but one case, these methods for membrane characterization are nondestructive and can be used repeatedly on a given membrane. These techniques provided the means for quantitative comparisons of immobilization methods and made possible temporal studies of immobilized enzyme activation.

Sensor Design - We complete the discussion of mathematical treatments of first generation amperometric enzyme electrodes by elaborating on some of the design aspects of sensor development. The detection limit and linearity of the sensor response are two aspects that play an important role in the design and optimization of amperometric enzyme electrodes. First, the limit of detection is controlled by three factors: the magnitude of the background current, the sensitivity, and the reproducibility. Sensitivity refers to the slope of the (response current-substrate concentration) calibration curve, di/dc_s, and is the only factor of the three that can be predicted by the previous models; thus, our discussion of detection limits is limited simply to sensitivity.

The preceding discussion of the work of Mell and Maloy [2] and Bartlett and Whitaker [3] indicate that the two key design factors which influence the sensitivity of an amperometric enzyme electrode are the enzyme loading and the thickness of the immobilized enzyme membrane. For a fixed enzyme loading, a decrease in the membrane thickness at low substrate concentration will yield an increase in the current and thus an increase in the sensitivity, provided the process is mass-transfer controlled. For a membrane of a given thickness, the slope of the calibration curve (and by definition the sensitivity) will increase with the amount of enzyme added (yielding a large relative catalytic activity per unit area or loading factor, σ_s) until it becomes equal to the value dictated by mass transfer. High values of σ_s also can be advantageous in sensor design for extending the range of

linearity beyond the value of the intrinsic substrate Michaelis constant K_S. For example, an increase in the loading factor σ_S from 1 to about 100 results in a shift in the upper limit of linearity by almost a factor of 100. In summary, the one-substrate models show that imposing diffusional limitations on the enzyme reaction can lead to improvements in sensor performance by increasing the sensitivity to the substrate, extending the range of linearity, decreasing the sensitivity to enzyme inactivation, as well as to reduce the time to reach steady state after a perturbation of the system.

A two-substrate enzyme-electrode model is a necessary guide in the design of sensors when the cosubstrate can become the limiting substrate. Because identification of the limiting substrate is of key importance in a cosubstrate-sensitive electrode, Leypoldt and Gough [5] give an expression for the critical relative catalytic activity. The practical significance of this parameter is in indicating the point at which the change from glucose to oxygen limitation occurs and at which the difference current becomes independent of glucose concentration. Therefore, application of their expression for the critical activity provides a powerful tool in designing practical sensors. Other model results [5] pertinent to sensor design include:

- The use of excess catalase (that maintains the activity of the glucose oxidase by destroying the damaging H_2O_2) *vs.* no catalase yields a two-fold increase in the detectable glucose range.

- An increase in the membrane permeability ratio of oxygen relative to the substrate increases the detectable range in a direct proportion to this ratio.

An ideal situation is one in which a thin membrane favoring oxygen transport over glucose is employed, so that oxygen remains in excess within the reaction layer. In other words, an appreciable extension of the range of detectability is possible by appending a noncatalytic membrane of preferentially restricted glucose permeability adjacent to the enzyme membrane. The development of novel membranes for this purpose can be carried out independently, since the details of enzyme kinetics are relatively unimportant for sensors operating in the more desirable diffusion-limited mode.

Second Generation Sensors

In this section, we review the theoretical work in the literature that has been carried out to analyze second generation amperometric enzyme electrodes. Limited work to model this type of diffusing, synthetic mediator-based sensor has been reported to date. However, the theoretical framework for second generation enzyme electrodes is similar to that used for first generation sensors; the redox mediator simply replaces the O_2/H_2O_2 couple.

Senda *et al.* [9] report experimental and theoretical results for the electrocatalytic oxidation of D-gluconate at a ubiquinone/dehydrogenase-mixed carbon paste electrode. The dependence of the steady-state electrocatalytic current on the concentration of the substrate and mediator was described by a Michaelis-Menten kinetic equation, without accounting for concentration variations of either the substrate or mediator. The approach is stated to be valid since the σ modulus, the ratio of the rate of reaction to that of diffusion in the enzyme-immobilized layer, is small (less than 0.2). This requirement must apply to both the substrate and mediator. Finally, the kinetics of the process were analyzed using this simple algebraic model of the catalytic current at a modified enzyme electrode with a diffusing mediator, and the apparent kinetic parameters were determined.

In another paper, Senda *et al.* [10] present a model that is focused on the diffusion accompanied with enzyme reaction of substrate (D-glucose) and mediators (benzoquinone and its reduced form) in the immobilized-enzyme layer and the diffusion in the semi-permeable membrane. The amperometric response of the electrode is analyzed (similar to the approach that Gough and Leypoldt [4], [5] apply to first generation sensors) as a function of transport, kinetic, and geometrical parameters and is compared with experimental results.

Albery and Cranston [11] extend the earlier model of a third generation sensor [12] (to be discussed in the next section) to account for a homogeneous enzyme-mediator reaction in the internal solution compartment. Concentration variations within the electrolyte layer are neglected, and their simplified algebraic modeling approach has limited predictive ability.

Most recently, Bartlett *et al.* [13] have developed a second generation enzyme sensor for hydrogen peroxide and *m*-chloroperbenzoic acid. The enzyme, cytochrome *c* peroxidase, is immobilized on a nylon membrane, and the diffusing homogeneous mediator, 1,3′-dimethylferrocene ethylamine, is reduced at the underlying gold rotating disk. The model that is used to characterize the sensor response is essentially identical to that reported in reference [3] for their first generation enzyme immobilized electrode. The difference being that the reaction-diffusion equation for the mediator is used in the place of equation (1) for hydrogen peroxide. Excellent agreement is obtained between experiment and theory.

Third Generation Sensors

Third generation amperometric enzyme electrodes provide a simple and direct approach for sensing because electrical communication between the redox enzyme (or modified enzyme) and the electrode is achieved without the use of diffusing mediators. In this section, we shall briefly discuss a third generation sensor that utilizes this relatively simple (in concept) direct electron-transfer reaction scheme. A schematic of the (hypothetical) sensor based on non-mediated direct electron transfer between the enzyme and electrode is given in figure 2 illustrating the overall detection process, where the enzyme (or modified enzyme), E_{ox}, first selectively oxidizes its substrate, S, (while simultaneously being reduced), yielding the reaction product, P, and E_{red}; the reduced form of the enzyme (or modified enzyme) is then directly oxidized at the electrode, and the resulting anodic current is a direct measure of the amount of substrate present in the solution. For this particular sensor configuration, a membrane of thickness L_m and permeable to the substrate and product confines the enzyme solution to a small (reaction) compartment of thickness L adjacent to the detection electrode.

Albery and Bartlett [12] have developed relatively simple mathematical models that describe this particular third generation enzyme electrode illustrated in figure 2. Two approaches are presented. The first is an algebraic model, where the internal solution or reaction zone is assumed to be thin enough (a few microns) that concentration variations of all solutes within the layer can be neglected.

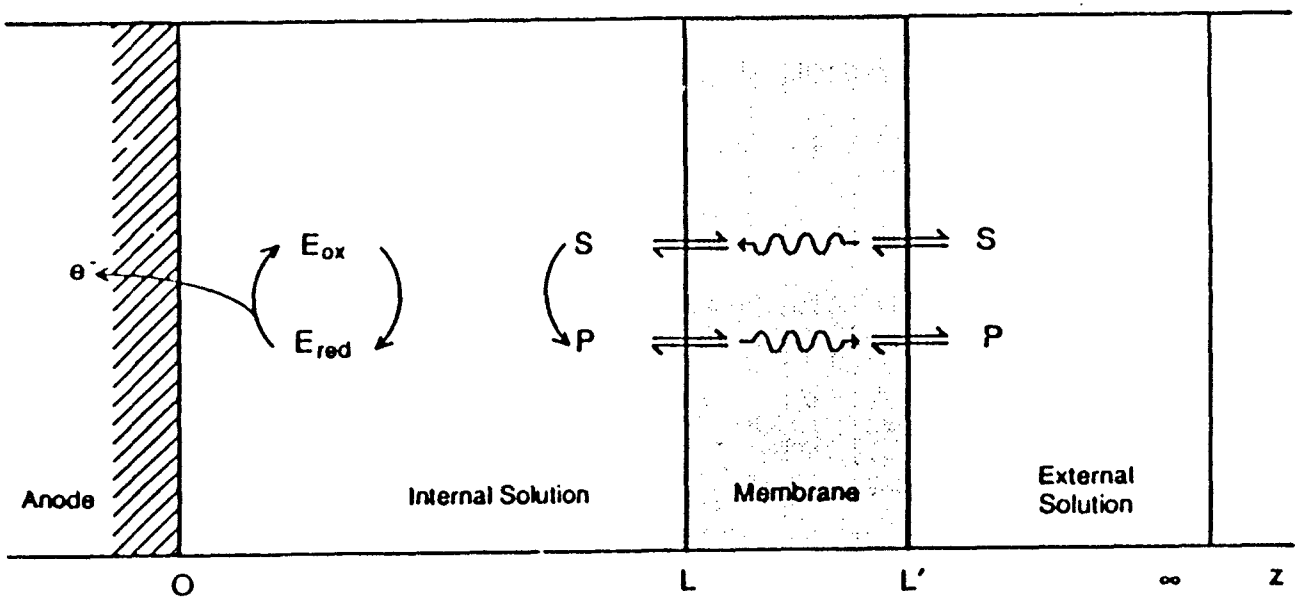

Figure 2. Hypothetical third generation amperometric sensor based on non-mediated direct electron transfer between the enzyme (or electrically modified enzyme) and electrode.

In the second approach, concentration variations of the enzyme are properly accounted for, but a simplified reaction mechanism for the enzyme is used. The latter assumption implies that the substrate and product can be excluded from the resulting single differential-equation model. Let us discuss each approach in more detail.

The first model accounts for diffusion of the nonenzyme solutes through the membrane and partitioning at the solution-membrane interfaces. The following linearized form of Fick's law for species i = S and P is used to describe the flux across the membrane

$$N_i^{(m)} = -h_{i,m}(\bar{c}_{i,\infty} - \bar{c}_{i,0}) \tag{17}$$

where the mass transfer coefficient for species i in the membrane is given by $h_{i,m} = \alpha_i D_i / L_m$. Because external mass-transfer effects are assumed to be negligible (due to the well-stirred external solution), equation (17) would typically serve as a boundary condition to the problem. However, concentration gradients are not being accounted for in the internal (reaction) compartment. Therefore, integration of the the steady-state material balance ($\int \nabla \cdot N_i = \int_0^L R_i$) and subsequent substitution of the production rate R_i due to chemical reaction in the bulk of the solution yields

$$N_i^{(int)} = -L \sum_l \nu_{i,l} \hat{R}_l \tag{18}$$

for species i = S, P, E_{ox}, and E_{red}. The rate, $\hat{R}_l$, of the homogeneous reaction l must be specified next.

The enzyme catalyzed conversion of substrate to product, shown in the figure, is assumed to proceed by the following reaction mechanism:

$$S + E_{ox} \rightleftharpoons E{\cdot}S \rightleftharpoons E{\cdot}P \rightleftharpoons E_{red} + P \tag{19}$$

where E·S and E·P are enzyme reaction intermediates. The expression for the rate of each of the three individual reaction steps is given by

$$\hat{R}_l = \hat{k}_l \prod_i \bar{c}_i^{\nu_{i,l}} - \hat{k}_{-l} \prod_i \bar{c}_i^{-\nu_{i,l}} \quad , \tag{20}$$

where $\bar{c}_i$ is the concentration of species i uniformly distributed in the internal solution compartment and $\nu_{i,l}$ is the stoichiometric coefficient of species i in reaction l. The forward and backward rate constants, given by $\hat{k}_l$ and $\hat{k}_{-l}$, respectively, are either first or second order.

The final governing equation used in the algebraic model of Albery and Bartlett [12] typically serves as the electrode boundary condition. Equation (15) for the flux of an electroactive species reduces to

$$N_i^{(0)} = -s_i r = -s_i k' \bar{c}_{E_{red}} \tag{21}$$

for $i = E_{red}$ and E_{ox}. The rate of the single reaction, the oxidation of the redox center of the enzyme directly at the modified electrode

$$E_{red} \rightarrow E_{ox} + e^- \ , \tag{22}$$

is given by $r = i/F = k' \bar{c}_{E_{red}}$ and k' is the potential dependent irreversible heterogeneous rate constant.

Finally, the appropriate steady-state fluxes given by equations (17), (18) and (21) can be equated. After elimination of $\bar{c}_{S,0}$, $\bar{c}_{P,0}$, and the four enzyme concentrations (using $\bar{c}_{E_{tot}} = \bar{c}_{E_{ox}} + \bar{c}_{E:S} + \bar{c}_{E:P} + \bar{c}_{E_{red}}$) the resulting expression for the reciprocal of the rate of reaction (or flux), $1/r$, is a function of the bulk concentrations $\bar{c}_{S,\infty}$ and $\bar{c}_{P,\infty}$, the various rate constants and mass-transfer coefficients and r. Limiting cases of the final reciprocal expression are discussed in terms of the different possible rate-determining processes. A simple diagnostic plot is applied for this purpose. Additionally, the effects of inhibition by the accumulation of product behind the membrane are considered.

In the second approach to characterizing the steady-state operation of a third generation amperometric enzyme electrode, Albery and Bartlett [12] use the following simplified reaction mechanism

$$E_1 \rightleftharpoons E_2 \quad \text{and} \quad E_2 \rightarrow E_1 \ , \tag{23}$$

where the first step is a homogeneous enzyme reaction and the second is the heterogeneous electrode reaction. The governing equation

$$D_i \nabla^2 c_i = \nu_i \hat{R} \tag{24}$$

for $i = E_1$ and E_2, where $\hat{R} = \hat{k}_f c_{E_1} - \hat{k}_b c_{E_2}$, can be solved with the electrode boundary condition

$$N_{i,0} = -D_i \nabla c_i = -i/F = -k'' c_{E_2} \tag{25}$$

yielding the final explicit expression for the reciprocal of the current density.

Parameter Determination

In order to effectively design and improve biosensor systems, it is necessary to establish methods for evaluating the various phenomena that are important in sensor operation. We have presented mathematical treatments of amperometric enzyme electrodes that account for substrate and product mass transfer, partitioning of solutes between the membrane and solution, enzyme reaction kinetics, and the electrochemical detection process. In this subsection, the emphasis is placed on analysis of the kinetic behavior of immobilized enzymes coupled with the simultaneous effect of diffusional resistances and partitioning. In order to facilitate the theoretical treatment of this phenomena for the purpose of parameter determination, the terminology proposed by Engasser and Horvath [14] is reviewed. The following definitions of kinetic rates and parameters are necessary for distinguishing among the different factors that affect the kinetics of the bound enzymes. The true kinetic behavior of an immobilized enzyme is characterized by its intrinsic kinetic parameters. Intrinsic kinetics are in effect only when no partitioning or diffusional effects are present. It is the intrinsic kinetics and rate parameters of the immobilized enzyme that are most useful for comparing the results of different immobilization procedures, but which are rarely accessible experimentally. The inherent kinetics prevail when partitioning, but not diffusional, effects are present. The apparent or effective kinetics are observed when internal or external diffusion effects are present.

It is common to determine effective kinetic parameters of immobilized enzyme systems by applying adaptations of the classical principles of enzymology developed for soluble enzymes that do not consider mass transfer. For example, Lineweaver-Burk and Eadie-Hofstee plots, both based on transformations of the Michaelis-Menten expression, have been used to extract apparent catalytic and Michaelis constants of the immobilized enzyme from the intercepts and slopes of these plots. However, it has been recognized that such plots often do not give straight lines when applied to immobilized enzymes [14], [15] because of the internal and/or external mass-transfer limitations. Extrapolation of apparently linear regions of these plots therefore do not lead to parameter estimates of clear physical significance. This approach gives effective kinetic parameters, which are valid only for

a particular set of experimental conditions, rather than more generally applicable intrinsic kinetic parameters. Despite these significant shortcomings of the Lineweaver-Burk and Eadie-Hofstee plots, let us discuss each in more detail because of their continued popularity.

If the effects of mass transfer are neglected, then the Michaelis-Menten kinetic expression [equation (2)] can be rearranged to give the following electrochemical Lineweaver-Burk equation:

$$\frac{1}{i} = \frac{1}{i_{max}} + \frac{K_S}{i_{max}} \frac{1}{c_{S,\infty}} + \frac{K_{O_2}}{i_{max}} \frac{1}{c_{O_2,\infty}} \tag{26}$$

where $i_{max} = n F L V_{max}$. Under conditions of no oxygen-reaction limitations ($K_{O_2} \rightarrow 0$), a plot of $1/i$ *vs.* $1/c_{S,\infty}$ should yield a straight line, if the substrate reaction is the rate-controlling process. Thus, the intercept of this plot yields $1/i_{max}$, and the slope gives K_S/i_{max}. Nonlinear behavior occurs when diffusional limitations are significant, and the slope of the curve depends upon the value of the Thiele modulus and the ratio of the Michaelis constant to the surface substrate concentration [15]. At relatively low substrate concentrations ($c_{S,\infty} \ll K_S$), the Lineweaver-Burk plot is asymptotic to a straight line, the slope of which is much steeper than that obtained in the absence of diffusional influences. With increasing substrate concentration, the plot becomes concave with respect to the abscissa because of the influence of diffusion on the zero-order character of Michaelis-Menten kinetics. The range of substrate concentrations over which this curvature is apparent increases with increasing Thiele modulus. At very high substrate concentration ($c_{S,\infty} \gg K_S$), the plot becomes asymptotic to the straight line corresponding to diffusion-free kinetics. The presence of an external diffusion resistance diminishes the concave curvature and increases the slope of the plot. Again, these results indicate that considerable caution should be exercised in the use of Lineweaver-Burk plots with immobilized enzymes because indiscriminate use may lead to kinetic parameter estimates which have little or no physical significance.

The other commonly used diagnostic transformation of the Michaelis-Menten equation is the Eadie-Hofstee plot. Again, assuming no mass-transfer limitations, the one-substrate enzyme kinetic expression can be rewritten as

$$i = i_{max} - K_S \left[\frac{i}{c_{S,\infty}} \right] \quad . \tag{27}$$

Thus, a plot of i *vs.* $i / c_{S,\infty}$ should produce a straight line with an intercept of i_{max} and a slope of $-K_S$. Although this plot has only limited applicability for parameter determination for the reasons stated above, it has been found particularly appropriate for the diagnosis of external and internal diffusional resistances [14]. The deviations from linearity due to diffusional effects are more pronounced and are easier to discern on this plot than on the Lineweaver-Burk plot. Additionally, Eadie-Hofstee plots can yield information about the nature of the diffusional effect, since external and internal limitations manifest themselves in concave and sigmoidal curves, respectively.

The popularity of the Lineweaver-Burk plot has remained because this form of the Michaelis-Menten expression allows the separation of the various resistances, that reduce the total current from its maximum possible value i_{max}. For example, in equation (26), the substrate and oxygen kinetic resistances are separated. Additionally, mass-transfer effects, as well as other types of resistances, are commonly added to equation (25) (although mass-transfer effects were originally neglected in the derivation of the equation) yielding a reciprocal expression for the current density. Although this is an attractive procedure due to its simplicity and is advocated by some experts in the field [16], it shall be evident from the next paragraph that such an equation should be used for only limited circumstances.

An expression for the current density of an amperometric enzyme electrode was given by Gough *et al.* [4], and when equation (15) is rearranged, the reciprocal expression is given by

$$\frac{1}{i} = \frac{1 + Bi_P^{-1}}{i_d - \dfrac{\eta \phi^2 i_d}{Bi_S} + \dfrac{\eta \phi^2 i_d}{Bi_P}} \tag{28}$$

where $i_d = n F D_S \alpha_s c_{S,\infty} / L$. The effectiveness factor η is a complex function of Bi_S, ϕ, and K_S for a one-substrate sensor. Because mass transfer and kinetics are strongly interrelated in equation (28), these effects should not be separated since it would not be rigorously meaningful in the general sense. However, for certain limiting cases, a reciprocal relationship, where mass-transfer effects are simply added on, may be helpful for electrode design, but in no way does this over-simplified model have

predictive abilities.

Next, we briefly review techniques that employ rotating disks and rotating ring-disk electrodes for the determination of intrinsic kinetic parameters of immobilized enzymes electrodes, since concurrent evaluation of mass transfer is required. Gough *et al.* [17] introduced a novel RDE system for first determining the fundamental transport properties of hydrophilic gel membranes. Transient analyses were employed to evaluate the permeability, the partition coefficient, and the diffusion coefficient in membranes, where the overall process is either strictly diffusion limited or partially electrode-reaction limited. In the latter case, the analysis is useful for estimating heterogeneous reaction-rate constants. Once the mass-transfer parameters that describe internal and external diffusion are determined, one can then proceed with the estimation of intrinsic enzyme kinetic parameters. The determination of the catalytic activity of immobilized glucose oxidase is complex because two substrates are involved, and numerical solutions to the governing differential equations that take into account mass transfer are required. Finally, rotating ring-disk enzyme electrodes also have been developed and evaluated [18]. Again, this particular electrode geometry is not being suggested for use as a practical biosensor, but instead, the immobilized enzyme RRDE system is employed for the purpose of characterizing mass transport and reaction kinetic parameters.

Summary

Mathematical models of amperometric enzyme electrodes that have appeared in the literature have been summarized. The phenomenological models are based on fundamental electrochemical and biochemical governing equations that describe the coupled transport and bioelectrocatalytic processes. Such quantitative analysis is needed for both, gaining insight and further understanding of the biosensor system, and for clarifying and explaining the interplay of the many factors that contribute to the electrochemical response of the sensor. Additionally, theoretical studies make it possible to discover new and better ways of plotting the experimental results for efficient data reduction and fundamental parameter determination. Finally, this approach should suggest directions for the engineering design and optimization of practical bioanalytical devices.

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